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TITLE: Zebrafish Functional Genetics Approach to the Pathogenesis of Well-Differentiated Liposarcoma

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14. ABSTRACT

Well-differentiated liposarcoma is the most common human sarcoma, and exposure to herbicidal agents used during the Vietnam war is a key predisposing factor. However, the pathobiology of this disease is very poorly understood, and there are no effective therapies for patients with unresectable disease. Almost all tumors have amplifications of multiple genes located on chromosome 12q, but the lack of animal models previously made it impossible to define the key "driver" oncogenes within these amplicons. Our overarching hypothesis is that defining the oncogenes driving selection for 12q amplifications in well-differentiated liposarcoma will reveal highly productive molecular targets for therapeutic intervention. During Year 1 of this award, we performed all work proposed under Aim 1 of our original proposal to test the hypothesis that FRS2 (fibroblast growth factor receptor substrate 2) is a 12q oncogene that activates oncogenic signal transduction, using FRS2 overexpression in genetically engineered zebrafish models and in normal human preadipocytes. Work during the upcoming year will focus on Aim 2 of our original proposal, testing the hypothesis that MDM2, CDK4 and HMGA2 are key liposarcoma oncogenes, and on developing novel therapeutic strategies for patients with these tumors, which are highly resistant to conventional therapy.

15. SUBJECT TERMS
Well-differentiated liposarcoma, oncogene, zebrafish, genetically engineered animal models, cancer therapy

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Zebrafish Functional Genetics Approach to the Pathogenesis of Well-Differentiated Liposarcoma

1. INTRODUCTION:

Well-differentiated liposarcoma is the most common soft-tissue sarcoma of humans, and predisposing factors include exposure to dioxin-containing herbicidal agents used during the Vietnam War (Agent Orange) and radiation from Atomic Bombs (Institute of Medicine 1994; Kogevinas 1997; Preston 2007). The molecular pathogenesis of this disease is very poorly understood, and there are no effective medical therapies for patients whose tumors cannot be fully removed at surgery (Dalal 2008). Current knowledge of well-differentiated liposarcoma pathobiology has been driven by descriptive analyses of human tumors, which have revealed that almost all cases harbor amplifications of chromosomal material from 12q (Suijkerbuijk 1994; Pedeutour 1999). These amplicons consistently involve the MDM2, CDK4 and HMGA2 genes, which have long been hypothesized to play a pathogenic role in this disease. Several additional genes are also involved within these amplicons, some of which have also been proposed to be pathogenic. However, the lack of animal models of this disease has made it impossible to identify the key oncogenes that drive selection for these amplifications, and therefore are expected to represent the "Achilles' Heels" of this disease. Indeed, the products of such genes could offer optimal targets for therapeutic intervention, in contrast to non-pathogenic "passenger" genes which are amplified only because of their physical proximity to bona fide oncogenes. The difficulty of distinguishing driver from passenger genes has resulted in a dearth of functionally validated therapeutic targets, representing a major obstacle to the development of effective cancer therapies. We recently developed the first animal model of well-differentiated liposarcoma, induced by expression of a constitutively active AKT transgene in mesenchymal progenitors of p53-mutant zebrafish (Gutierrez 2011). Thus, this animal model now allows us to perform pioneering in-depth investigation of the molecular pathogenesis underlying well-differentiated liposarcoma.

2. KEYWORDS:

Well-differentiated liposarcoma, Oncogene, Zebrafish, Genetically engineered animal models, Cancer Therapy

3. ACCOMPLISHMENTS: Major Activities and Specific Objectives during the first year of this award are summarized here with respect to each task outlined in the approved Statement of Work, as requested:

3A. Specific Aim 1. Test the hypothesis that FRS2 overexpression drives aberrant PI3K-AKT activation in well-differentiated liposarcoma.

3A.1. Task 1. Test whether FRS2 is a well-differentiated liposarcoma oncogene in transgenic zebrafish
(timeframe, months 1-12).

Subtask 1a. Test whether FRS2 overexpression in zebrafish mesenchymal progenitors is sufficient to induce well-differentiated liposarcoma (timeframe, months 1-6). During year 1 of this award, we generated a cohort of zebrafish expressing a rag2:FRS2 transgene that drives FRS2 overexpression in mesenchymal progenitors. Positive controls were injected with rag2:myr-Akt2, and negative controls were injected with rag2:EGFP. In our original application, we proposed generating and testing a minimum of 50 zebrafish per condition, based on calculations of our biostatistician collaborator Dr. Kristen Stevenson. We analyzed a cohort of 81 rag2:FRS2 zebrafish, as well as 58 positive controls and 61 negative controls, for a minimum follow-up period of 6 months (range, 6-11 months), but we saw no liposarcomas develop in FRS2-transgenic animals (**Figure 1A**). Subtask 1a is now complete.

Subtask 1b. Test whether FRS2 overexpression in zebrafish mesenchymal progenitors collaborates with p53 mutation in well-differentiated liposarcoma pathogenesis (timeframe, months 3-9). We also generated a cohort of rag2:FRS2-transgenic zebrafish in the p53 homozygous mutant background, to test whether FRS2 overexpression collaborates with p53 mutations. We analyzed a cohort of 91 rag2:FRS2, p53-mutant zebrafish for tumor onset for a minimum of 6 months (range, 6-8 months), as well as a cohort of rag2:myr-Akt2 positive controls and rag2:EGFP negative controls (>50 fish in each group), but also saw no liposarcomas develop in FRS2-transgenic animals (**Figure 1B**). Subtask 1b is now complete.

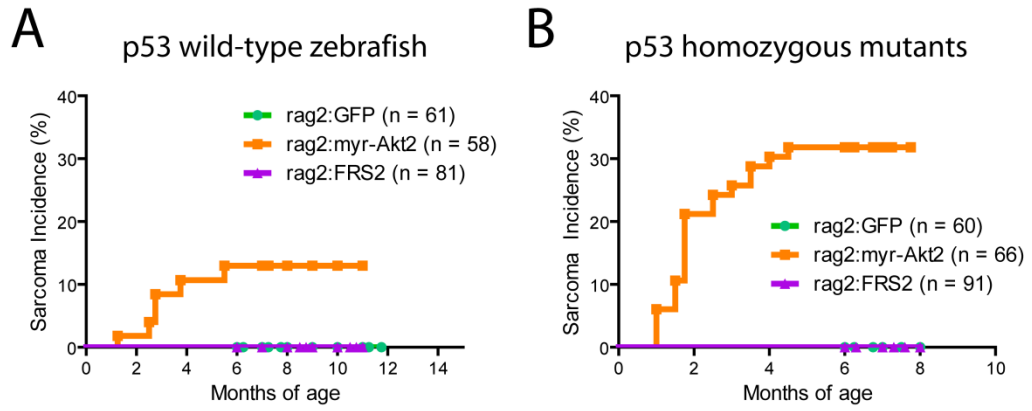

Figure 1. FRS2 is not sufficient to induce well-differentiated liposarcoma. To test whether FRS2 is a liposarcoma oncogene, we generated transgenic zebrafish in the p53 wild-type (A) or p53 homozygous-mutant background (B), which expressed rag2:FRS2, rag2:myr-AKT2 (positive control), or rag2:GFP (negative control) transgenes. This experiment revealed no tumors in FRS2-transgenic zebrafish indicating that FRS2 expression alone is not sufficient to drive liposarcoma development. By contrast, tumors in myr-AKT2 positive controls occurred at the predicted rate based on our previous findings (Gutierrez 2011).

Subtask 1c. Test whether FRS2 overexpression in zebrafish mesenchymal progenitors drives aberrant PI3K-AKT and RAS-MAPK pathway activation (timeframe, months 9-12). We was submitted a cohort 4 of zebrafish expressing rag2:FRS2, as well as rag2:myr-Akt2 (encoding constitutively active Akt2) and rag2:KrasG12D (encoding constituvitely active Kras) positive controls, and rag2:EGFP negative controls for immunohistochemical analysis of AKT, S6K,

MEK and ERK. Samples are currently being processed in the histopathology core facility, and we anticipate having results later this month.

3A.2. Task 2. Test whether FRS2 overexpression in normal human preadipocytes promotes proliferation and oncogenic signal transduction (timeframe, months 1-12).

For all experiments in this Task, normal human preadipocytes are from a commercial provider (www.promocell.com, product # C-12730). The human liposarcoma cell lines we will use are LPS 141, LPS 510, LPS 789, LPS 853, and T449, which were obtained from the laboratory of my co-mentor Dr. Jonathan Fletcher, where most of these lines were originally derived. Both normal preadipocytes and human liposarcoma cell lines will be provided to us anonymously without any linking identifiers, and it will be impossible for us to determine the identity of the original source.

Subtask 2a. Test whether FRS2 overexpression in normal human preadipocytes promotes proliferation and overcomes senescence (timeframe, months 1-6). We generated a lentivirus construct expressing full-length human FRS2, and used it to successfully infect normal human preadipocytes with this gene. EGFP was the negative control, and KRAS-G12D was the positive control. We then analyzed these cells for growth, proliferation, and apoptosis, but found no effect of FRS2 overexpression in these cells (**Figure 2**). Subtask 2a is now complete.

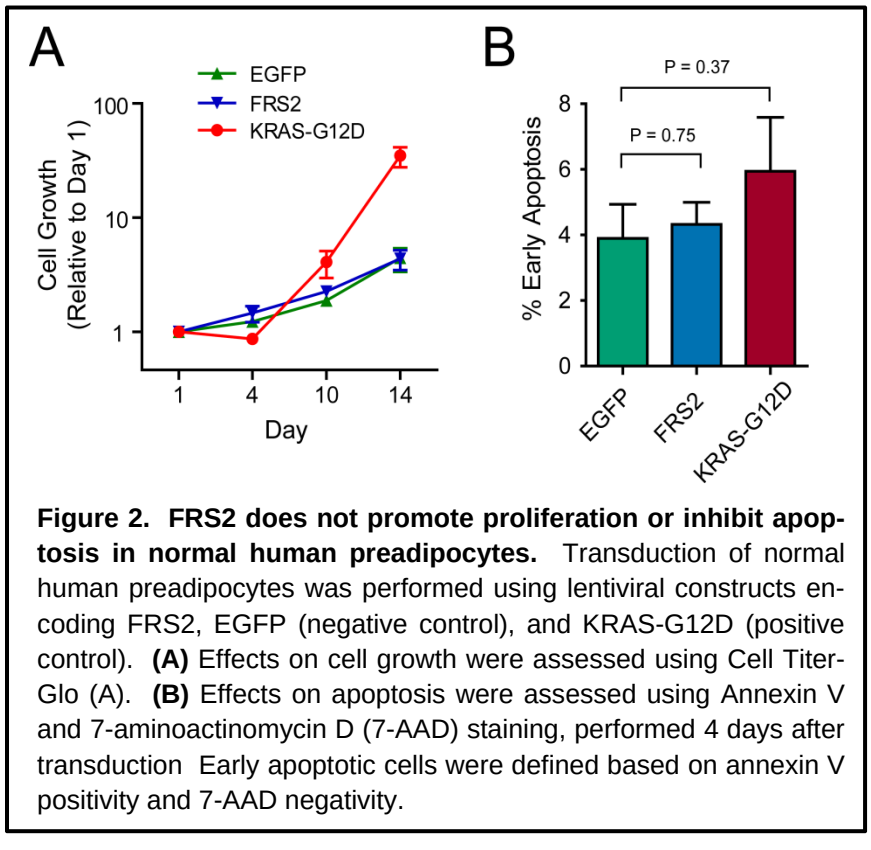

Figure 2. FRS2 does not promote proliferation or inhibit apoptosis in normal human preadipocytes. Transduction of normal human preadipocytes was performed using lentiviral constructs encoding FRS2, EGFP (negative control), and KRAS-G12D (positive control). **(A)** Effects on cell growth were assessed using Cell Titer-Glo (A). **(B)** Effects on apoptosis were assessed using Annexin V and 7-aminoactinomycin D (7-AAD) staining, performed 4 days after transduction Early apoptotic cells were defined based on annexin V positivity and 7-AAD negativity.

Subtask 2b. Test whether FRS2 overexpression in normal human preadipocytes drives oncogenic signal transduction (timeframe, months 3-9). We also analyzed the cells generated in Subtask 2a for biochemical evidence of activation of PI3K-AKT and RAS-MAPK pathways, using Western blot analysis for phosphorylation of AKT, S6K, MEK and ERK. This experiment revealed no evidence of activation of these signal transduction pathways **(Figure 3)** and this Subtask is now complete.

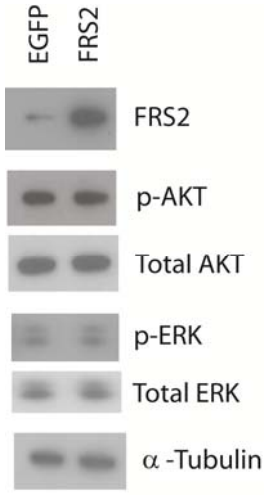

Figure 3. FRS2 does not induce activation of AKT or ERK signal transduction pathways. Normal human preadipocytes were transduced with FRS2 or EGFP (control), and activation of PI3K-AKT and RAF-MEK-ERK signal transduction pathways was assessed by Western blot for the indicated antibodies 4 days after transduction.

Subtask 2c. Test whether FRS2 is required for proliferation and survival of human liposarcoma cells (months 12-24). We performed shRNA knock-down in human cell lines derived from patients with well-differentiated liposarcoma that harbor amplification and overexpression of FRS2, and found that FRS2 knock-down specifically decreases cell viability and induces cell death through the mitochondrial pathway of apoptosis **(Figure 4)**. Subtask 2c is now complete.

3A.3. Task 3. Test the hypothesis that growth, survival, and oncogenic signal transduction in cell lines derived from patients with well-differentiated liposarcoma is dependent on FRS2 overexpression (timeframe, months 1-12)

Subtask 3a. Identify shRNA hairpins that effectively silence FRS2 expression in human liposarcoma cells in a conditional fashion (timeframe, months 1-3). We have identified two distinct shRNA hairpins that effectively silence FRS2 expression in human liposarcoma **(Figure 4A)**. Subtask 3a is now complete.

Subtask 3b. Test whether FRS2 knock-down in human liposarcoma cell lines impairs proliferation and oncogenic signal transduction (timeframe, months 3-9). We performed shRNA knock-down of FRS2, and found that this impairs proliferation and signaling through key downstream oncogenic signaling pathways, including the PI3K-AKT and MAPK pathways, as assessed by Western blot for phosphorylation of key downstream kinases including AKT, MEK and ERK. Subtask 3b is now complete.

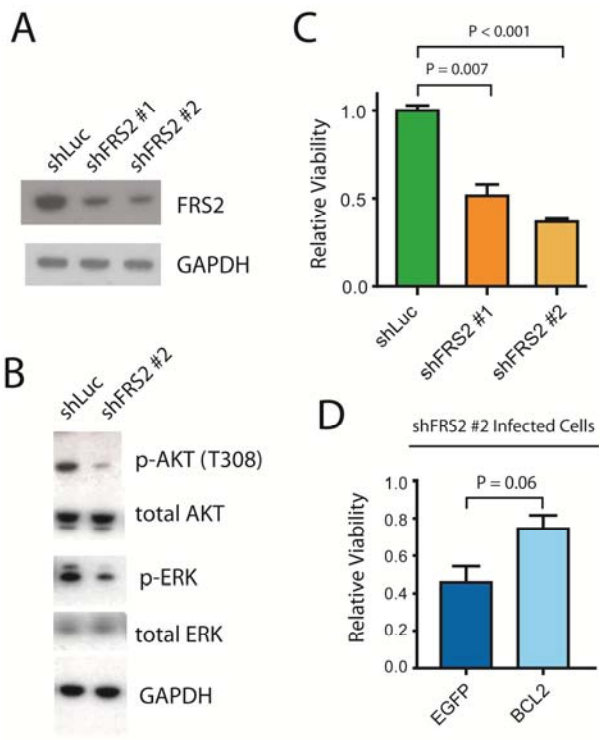

Figure 4. FRS2 knock-down impairs the viability of human liposarcoma cells. (A) Two individual shRNA hairpins were identified that effectively knock down FRS2 expression in LPS141 cells, a human liposarcoma cell line. Western blot analysis shown. (B) FRS2 knock-down silences activation of the AKT and ERK pathways, as assessed by Western blot analysis for the indicated antibodies. (C) FRS2 knock-down impairs the viability of LPS141 cells, as assessed using Cell Titerglo analysis. (D) The toxicity of FRS2 knock down is partially rescued by overexpression of a BCL2 construct that blocks mitochondrial apoptosis. These findings implicate mitochondrial apoptosis induction as one key mediator of the toxicity of FRS2 knock-down.

Subtask 3c. Investigate the hypothesis that FRS2-dependent signal transduction requires an activated kinase (timeframe, months 9-24). Experiments to test this hypothesis are underway, and include both genetic appraoches based on RNAi silencing of kinases known to interact with FRS2, and a biochemical approach based on immunoprecipitation to identify kinases that interact with FRS2 in human liposarcoma cells. These experiments are underway and Subtask 3c will represent a focus of investigation during year 2 of this award.

3B. Specific Aim 2. Test the hypothesis that MDM2, CDK4 and HMGA2 are well-differentiated liposarcoma oncogenes that collaborate synthetically in its molecular pathogenesis (timeframe, months 12-24).

3B.1. Task 1. Test the ability of MDM2 to accelerate the onset of AKT-induced well-differentiated liposarcoma (timeframe, months 12-18). We have now generated the MDM2 expression construct and validated that it can successfully drive MDM2 expression and inhibit function of the key MDM2 target (p53) in vivo in zebrafish mesenchymal progenitors. We are poised to begin this experiment now (month 12 of the award), and expect to have this completed by month 18 of the award period.

3B.2. Task 2. Test the hypothesis that CDK4 and HMGA2 are well-differentiated liposarcoma oncogenes (timeframe, months 18-24). We have completed the cloning of the expression constructs driving CDK4 and HMGA2, and have planned experiments to validate that these drive appropriate expression *in vivo* in zebrafish mesenchymal progenitors, and both of these genes

will be tested for their ability to accelerate onset of AKT-induced liposarcoma during months 18-24 of this award.

3B.3. Task 3. Test the hypothesis that YEATS4 is a non-pathogenic passenger gene within the liposarcoma 12q amplicons (timeframe, months 12-24). We have completed the cloning of the YEATS4 expression construct, and experiments to validate that this construct drives YEATS4 overexpression *in vivo* in zebrafish mesenchymal progenitors are being finalized. As soon as we have confirmed this, we will proceed to test whether YEATS4 overexpression can accelerate the onset of AKT-induced well-differentiated liposarcoma in genetically engineered zebrafish.

Significance of Results and Conclusions for Year 1: Our work in Year 1 has yielded key results that allow us to conclude, based on our work in Specific Aim 1, that FRS2 overexpression is not sufficient to induce aberrant oncogenic signal transduction in well-differentiated liposarcoma, thus our hypothesis that FRS2 is a strictly defined oncogene has been disproved. However, human liposarcoma cells are highly dependent on normal FRS2 function of FRS2, and we remain quite optimistic that identifying and therapeutically targeting the kinase required for FRS2-dependent signaling will yield an attractive therapeutic target. Indeed, increasing evidence is emerging in diverse tumor types that the targeting of key dependencies of cancer cells can represent highly effective therapies, even when the gene being targeted is not a bona fide oncogene, and we remain optimistic that our work in Specific Aim 1 during year 2 of the award will reveal this to be the case.

Drawing significance and conclusions from our work in Aim 2 will require ongoing investigation, which will be the major focus of our efforts during Year 2 of this award.

Opportunities for Training and Professional Development: This award has allowed me to formalize a mentoring relationship with my co-mentors on this project Drs. Thomas Look (Dana-Farber Cancer Institute) and Jonathan Fletcher (Brigham & Women's Hospital), and the opportunity to interact scientifically with both of these leading cancer biologists has provided me with invaluable mentorship and guidance as I establish my independent research program. Additionally, work in this proposal has been the focus of a student and a very talented research technician in my laboratory, and this project has represented an ideal training experience for both of these individuals as they continue their development into the next generation of physician-scientists.

How Were the Results Disseminated to Communities of Interest: A portion of our results were presented in an Oral Platform Presentation at the Third International Conference on Sarcoma Biology (New York, NY). Once our studies are complete, they will be submitted for publication in a peer-reviewed scientific publication.

What we plan to do during the next reporting period to accomplish our goals: We plan to achieve all of our originally proposed goals as described under each task as outlined in Section 3 above.

4. IMPACT: Our work during Year 1 of this two-year award is not yet sufficiently mature for its impact to be fully assessed, but I am confident that work that will be our focus during Year 2

of this award will lead to a major improvement in our understanding of the molecular pathogenesis of well-differentiated liposarcoma, as well as reveal novel effective therapeutic strategies for this common subtype of sarcoma, which is completely refractory to chemotherapy and radiation. Exposure to dioxin-based herbicidal agents (Agent Orange) and to radiation are known predisposing factors for this sarcoma, thus this work is expected to have significant future impact on Veterans with these tumors.

5. CHANGES/PROBLEMS: No changes are planned.

6. PRODUCTS:

Publications, conference papers, and presentations: A portion of this work was presented at the Third International Conference on Sarcoma Biology, New York, NY (2013). This presentation is noted in my curriculum vitae, included as Appendix I to this annual report.

7. PARTICIPANTS AND OTHER COLLABORATING INSTITUTIONS:

Participants:

Name: Alejandro Gutierrez
Project Role: Principal Investigator
Researcher Identifier: ORCID 0000-0002-0249-9007
Nearest person month worked: 2.4 months
Contribution to project: Dr. Gutierrez has been responsible for directing all aspects of this project. This includes designing experiments, performing technically challenging aspects of key experiments, and interpreting data and results.
Funding Support: Research grants from the National Institutes of Health/National Cancer Institute, USC Parker Institute, Gabrielle's Angel Foundation for Cancer Research, Damon Runyon Cancer Research Foundation, Linde Family Foundation, Boston Children's Hospital.

Name: Christine Reynolds
Project Role: Research Technician
Researcher Identifier: N/A
Nearest person month worked: 0.6 months
Contribution to project: Ms. Reynolds has been responsible for carrying out key technical aspects of this proposal, particularly with respect to cloning and testing of the constructs for Aim 2. Ms. Reynolds is also our lab manager and has been managing ordering and supplies for this project.
Funding Support: Research grants from the National Institutes of Health/National Cancer Institute, USC Parker Institute, Gabrielle's Angel Foundation for Cancer Research, Damon Runyon Cancer Research Foundation, Linde Family Foundation, Boston Children's Hospital.

Name: Oscar Calzada
Project Role: Research Technician
Researcher Identifier: N/A

Nearest person month worked: 12 months
Contribution to project: Mr. Calzada has been primarily respondible for the performance of all of the work in this proposal, with the assistance of Ms. Reynolds as detailed above. Mr. Calzada was also involved in designing the experiments and interpreting the results.
Funding Support: N/A

Changes in the Active Other Support of the PI:

1. Grants that have expired since start of the current award:

TITLE: **Zebrafish Chemical and Classical Genetics Approach to the Pathogenesis of T-ALL**
TIME COMMITMENT: 75%
AGENCY: NIH 5K08 CA133103-04
PERFORMANCE PERIOD: 07/01/08 – 06/30/13

TITLE: **Discovery and Targeting of Apoptosis Resistance Mechanisms in T-ALL**
TIME COMMITMENT: 10%
AGENCY: NIH 1R21 CA167124-01
PERFORMANCE PERIOD: 06/01/12 – 05/31/14

TITLE: **Unraveling the Molecular Pathogenesis of T-Cell Acute Lymphoblastic Leukemia using Zebrafish Genetics and Small Molecule Screens**
TIME COMMITMENT: 10%
AGENCY: American Society of Hematology
PERFORMANCE PERIOD: 07/01/08 – 06/30/13
LEVEL OF FUNDING: $104,140 (annual direct costs)

2. New grants received since start of the current award:

TITLE: **Therapeutic Activation of the PP2A Tumor Suppressor for High-Risk T-ALL**
TIME COMMITMENT: 20%
AGENCY: USC Parker Institute
PERFORMANCE PERIOD: 01/01/13 – 12/31/14
OVERLAP: NONE.

TITLE: **Therapeutic Activation of the PP2A Tumor Suppressor in High-Risk T-cell Acute Lymphoblastic Leukemia**
TIME COMMITMENT: 0%
AGENCY: Gabrielle's Angel Foundation
PERFORMANCE PERIOD: 05/30/13 – 05/29/16
OVERLAP: NONE.

TITLE: **Molecular mechanisms of chemotherapy resistance in T-cell acute lymphoblastic leukemia**
TIME COMMITMENT: 5%
AGENCY: Boston Children's Hospital
PERFORMANCE PERIOD: 07/01/13 – 06/30/15
OVERLAP: NONE.

TITLE: **Mechanisms and Therapeutic Targeting of EZH2-Dependent Chemoresistance in T-ALL**
TIME COMMITMENT: 20%
AGENCY: Damon Runyon Cancer Research Foundation
PERFORMANCE PERIOD: 07/01/13 – 06/30/17
OVERLAP: NONE.

TITLE: **The role of JAK3 mutations in T-ALL**
TIME COMMITMENT: 10%
AGENCY: Linde Family Foundation
PERFORMANCE PERIOD: 06/01/14 – 05/31/15
OVERLAP: NONE.

8. SPECIAL REPORTING REQUIREMENTS: Not applicable.

9. APPENDICES:

Appendix I contains an updated curriculum vitae of the principal investigator, and highlighted in yellow is the presentation of a portion of the work funded by this award at the Third International Conference on Sarcoma Biology, New York, NY (2013).

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